

**Morphological divergence among
sympatric demes of European grayling
(*Thymallus thymallus*).**

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Forord (preface)

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Kim Magnus Bærum

Abstract

Divergent selection pressures, induced by variations in environmental conditions, can be expected to favour different phenotypic expressions. Different populations show thus often local adaptations that could be attributed to their respective environments. Such local adaptations have even been demonstrated to occur at a contemporary time scale. Observing rapid local adaptations in the wild may shed more light on speciation and fundamental evolutionary processes. In this study, I have explored morphological variation among demes of European grayling (*Thymallus thymallus*) within a Norwegian lake, Lesjaskogsvatnet. The morphological variation has been quantified using geometric morphometric methods. The grayling inhabiting the lake shared common ancestors less than 25 generations ago. I hypothesised there to be a divergence in morphological traits affecting survival and reproduction due to variations in sexual and natural selection within the system. I found differences in sexual dimorphic traits among demes. Further, I found a small difference in the degree of sexual dimorphism between demes. This morphological pattern may be due to among-tributary differences in environmental conditions during spawning, and thus different natural and sexual selection gradients. The among-deme difference may also have occurred due to founder effects and genetic drift. I also found evidence of polymorphism, which might indicate there to be variations in resources or resource competition between different basins in the lake. All this morphological variations might have occurred due to either adaptive divergence or phenotypic plasticity.

Introduction

Different phenotypic traits are often favoured under different environmental conditions (Futuyma, 2005). As a consequence, populations inhabiting dissimilar environments may deviate in fitness-related traits. Divergence of such traits may occur due to phenotypic plasticity, local adaptations (evolution), or a combination of both. *Phenotypic plasticity* is referred to as the interaction between the environment and the expressed phenotype, without genetic change (Scheiner, 1993). The relationship between the environment and the phenotype is termed the trait's "norm of reaction". Many such reaction norms are affected by irreversible "developmental switches" occurring during an organism's ontogeny (Futuyma, 2005). *Local adaptations* (with genetic change) through selection are expected to happen when a population is moved away from a local fitness peak in an adaptive landscape (Fisher, 1930). The selection driving the among-populations difference could be of natural or sexual origin, or a combination of both (e.g. West-Eberhard, 1983; Taylor, 1991; Vantienderen, 1991; Schluter, 1994; Lu and Bernatchez, 1999; Hendry, 2001; Hoekstra et al., 2001; Schluter, 2001). In sexual selection, it may be sensible to separate even further between intra- (often competition among males) and inter- (sexual conflicts and female choice) sexual selections as driving forces. Intra-sexual competition often favours traits like "weapons" (examples: deer antlers and hooknose in salmon) in the fight for a mating partner(s). Inter-sexual competition could favour traits like exaggerated ornaments (examples: the extravagant tail of a peacock and enlarged, ornamented tailfins in guppies). Local adaptation to different environmental conditions has been documented numerous times and the process is even observable at a contemporary time scale (e.g.: Hendry and Kinnison, 1999; Huey et al., 2000; Bone and Farres, 2001; Haugen and Vøllestad, 2001; Grant and Grant, 2002; Svensson and Gosden, 2007). Such rapid evolution of diverging phenotypic traits is often associated with a novel host or food resource, a new biophysical environment, a new predator community, or a new coexisting competitor (reviewed in Reznick and Ghalambor, 2001).

Phenotypic difference among populations may also represent a more random factor: *Genetic drift* (e.g. Lande, 1976). Such trait divergence should however be recognised as neutral, with no fitness consequences related to the different environments. However, the relative contribution to phenotypic difference by genetic drift is likely to increase with

decreasing population size, with small effective size, and in bottlenecked populations (Lande, 1976; Carson and Templeton, 1984; Lynch, 1990). The latter are also conditions that are expected to constrain selection.

The majority of the examples found of contemporary evolution seem to be the result of natural selection and related trade-offs, and to a lesser degree sexual selection itself (Svensson and Gosden, 2007). This is despite the observation that sexual selection gradients seem to be more pronounced than natural selection gradients (Hoekstra et al., 2001; Kingsolver et al., 2001). What also seems evident from the literature is that the morphology of an individual is often directly, and strongly, linked to fitness (Koehl, 1996). Morphological traits, affecting both survival and reproduction, are thus frequently observed to be under strong selection (Kingsolver et al., 2001; Kingsolver and Pfennig, 2007) and may diverge rapidly between populations (e.g. Hendry, 2001; Grant and Grant, 2002; Eberhard, 2004; Collyer et al., 2005). It is, however, not fully known how, and where, contemporary evolution of traits between populations ultimately will result in speciation. Hence, in order to better understand the cause of contemporary adaptive evolution in nature, and its role in speciation processes, further explorations of morphological divergence, occurring on small spatial scales, should be conducted.

Salmonid study systems hold numerous benefits in the study of evolutionary processes, and have thus provided key insights on many evolutionary questions (reviewed in Hendry and Stearns, 2004). One of the greatest advantages is the long history of studying the phenotypic diversity within the family. This leaves a great deal of knowledge on the possible outcome of differential selection pressures, trait utility and diversifying factors. This enables comparisons between systems and, hence, greater insight in the wide range of diversifying processes and adaptive radiations in the wild.

The study system in the lake Lesjaskogsvatnet (Norway) is remarkable in many ways. European grayling (*Thymallus thymallus*) colonised the lake in the 1880s. During a short period, they migrated freely through a man-made and temporary channel connecting the upper part of Gubrandsdalslågen and Lesjaskogsvatnet. This means that all grayling within the lake share a recent common ancestry some 20-25 generations ago (Haugen and Vøllestad, 2001). Grayling are now found throughout the lake. It is, however, not yet known if the fish moves freely all over the lake, or if they are constrained to different basins within the lake.

Irrespective of the latter, the lentic grayling spawns in many of the tributaries connected to Lesjaskogsvatnet (Gregersen, 2005), and signs of reduced gene flow and genetic structuring has been observed between the grayling in the different spawning tributaries (demes) (Barson et al., 2008). Many of these tributaries differ in environmental conditions like temperature, flow regime and size, which may thus expose the various demes to different selection pressures during spawning (one to two weeks in spring time) and early ontogeny. The study system have indeed recently provided evidence of contemporary evolution of life history traits in grayling that can be attributed to differential tributary environments (Gregersen et al., 2008). Further, closely related grayling populations in neighbouring lakes (introduced 12-22 generations ago, with common ancestry in Lesjaskogsvatnet) have also shown rapid evolution and divergence rates on important early life-history traits - despite signs of severely bottlenecked populations (Haugen, 2000; Haugen and Vøllestad, 2000, 2001; Koskinen et al., 2002). Given the possible strong selection on reproductive traits and morphology affecting fitness (Kingsolver et al., 2001; Kingsolver and Pfennig, 2007), and variations in environmental conditions between spawning tributaries, one might also expect to observe among-deme variations in grayling morphology. Hence, in this thesis I will explore and quantify morphological divergence between grayling demes (tributaries) in Lesjaskogsvatnet. This study is a part of a larger ongoing project in the study system: “The early stages of adaptive radiation: sympatric divergence in grayling”.

Four tributaries were chosen within Lesjaskogsvatnet: Two small and warm tributaries (SW-tributaries) and two large and cold tributaries (LC-tributaries). SW-tributaries may offer fewer suitable spawning sites in relation to number of spawning grayling, and spawning individuals may thus here experience high densities. There are also, in general, less visual obstacles in the SW-tributaries which may result in male grayling demanding larger territory size (Northcote, 1995). Hence, the strength of sexual selection is hypothesised (H1) to be stronger in the SW-tributaries, which here should favour morphological traits enhancing male performance at the spawning ground. Such secondary sexual characters in salmonid are typically exaggerated jaws (e.g. Quinn and Foote, 1994) and large dorsal hump size (e.g. Berg, 1979). It is to my knowledge not yet known if these secondary sexual characters are present in grayling, but the large and colourful dorsal fin seen in males has been recognised as important in the spawning act (Fabricius and

Gustafson, 1955; Kratt and Smith, 1980). The LC-tributaries have, during the spawning period, a more turbulent flow compared to the more laminar flow experienced in the SW-tributaries. This could influence the drag experienced by individuals in the different tributaries. A turbulent flow could impose a more energy demanding spawning migration (e.g. Hinch and Rand, 1998). Streamlining reduces however the net drag (Koehl, 1996). Hence, a more streamlined body could hypothetically (H2) be favoured by natural selection when migrations are more demanding (e.g. Blair et al., 1993; Hendry and Berg, 1999; Hendry, 2001; Kinnison et al., 2003). There might be differences in nutrition and prey composition among basins in the lake (Gammelsrud, 1982). However, all the grayling can, in theory, freely swim around the entire lake in non-spawning periods. Hence, I expect various possible selection agents within the lake to have a uniform effect on all the grayling (H3).

Based on the three hypotheses (H1-H3), I predict the following:

H1:

- P1: Male grayling in the SW-tributaries possesses more exaggerated sexual characters compared to males from the LC-tributaries.
- P2: Sexual dimorphism, concerning morphological traits, is more pronounced in the SW-tributaries demes compared to the LC-tributaries demes.

H2:

- P: Grayling in the LC-tributaries possesses a more streamlined body (i.e. reduced body depth) compared to the grayling in the SW-tributaries.

H3:

- P: There should be no morphological divergence which could attributed to a basin effect.

Material and methods

The grayling

A general review of the grayling (*Thymallus* spp.) life cycle is available in Northcote (1995). The European grayling typically thrives in oxygen rich and clean freshwater systems throughout Europe. In Norway, grayling is naturally distributed in the northern part of the country and in larger river systems in the south-eastern part. The grayling has also been introduced to adjoining lakes and smaller river systems, as in the study system for this thesis. Grayling starts their spawning run in springtime, after ice-out, when mean water temperatures exceed 4–5 °C in the spawning areas. Mature lentic grayling then return to their natal stream with high precision (Kristiansen and Døving, 1996). The spawning grayling stays in the stream for a short period, where it shows an extensive and complex courting behaviour (see Fabricius and Gustafson, 1955). The grayling is atypical in that males defend and hold territories, and females approach and deposit their eggs in the male's "nest" (Northcote, 1995). The males show a strongly increased aggressive territorial behaviour, often with subsequent fighting among individuals, while guarding their spawning ground. Ability to effectively manoeuvre in the current, size and stamina seem to be important traits during these male to male encounters. However, sneaking acts by non-territorial small males during spawning between females and larger males have also been observed (Kratt and Smith, 1980; Poncin, 1996). The large and coloured dorsal fin plays seemingly a very important role in the male sexual display, and males are indeed an impressive and eye-catching sight when showing the full extent of their fins. The females do also have fairly large dorsal fins, although not as impressive, colourful and large as the males.

Study area

The fish were collected in tributaries to lake Lesjaskogsvatnet (611 meter above sea level, surface area 4.52 km²; Figure 1) which is located in Lesja municipality, Oppland County, Norway. The lake consists of three shallow (mean depth 10 meter) continuous basins, which all are drained by two major outlets; Gubrandsdalslågen (flowing east) and Rauma (west). Besides grayling, the fish community consists of brown trout (*Salmo trutta*) and

European minnow (*Phoxinus phoxinus*). The general climate in the area during the year is dry and relatively hot during the summer (around June-August), and cold with relatively much precipitation (i.e. snow) during winter time (around November-March/April).

The lake is situated in a U-shaped valley, where positioning of the valley (west to east) makes the one side (north) more sun exposed and warmer than the other side. Due to this general temperature divergence and variation in the timing of spring snow melting, the various tributaries are found to be very different in flow velocities, temperatures and also in mean width (Gregersen et al., 2008). In a k-mean cluster analysis between tributaries performed by Gregersen et al. (2008), using tributary mean width and tributary June-August temperature sum as clustering covariates, two distinct cluster groups appeared:

- 1. Small and warm tributaries (SW-tributaries)
- 2. Large and cold tributaries (LC-tributaries)

The SW-tributaries are mainly found at the warmer north side, while the LC-tributaries are mostly found on the colder south side (Figure 1). With steeper hillsides, closer to the lake, the tributaries at the south side also tend to be in general faster flowing compared to the tributaries at the north side. The LC-tributaries are in general large (i.e. wide and/or in general “deep”) throughout the year, while the SW-tributaries can almost dry up and be reduced to a minimum during hot and dry summers (personal observation).

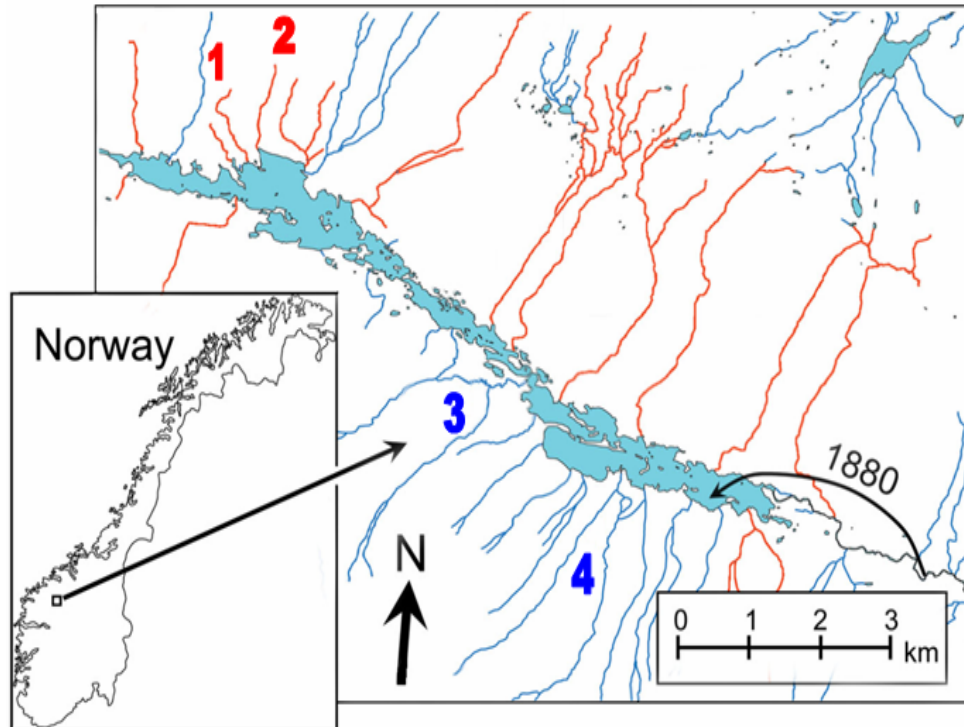


Figure 1: Map of Lake Lesjaskogsvatnet. Grayling colonized the lake in the 1880s from Gubrandsdalslågen (east outlet) and have since then established over 20 sub-populations in the different tributaries to the lake. Tributaries marked in red are typical SW-tributaries (Søre Skotte = 1, Steinbekken = 2), while blue are LC-tributaries (Hyrjon = 3 and Valåe = 4).

Four tributaries were chosen for the study, two SW-tributaries (Søre Skotte and Steinbekken) and two LC-tributaries (Hyrjon and Valåe) (Figure 1; Table 1).

Søre Skotte and Steinbekken are typically warm and small tributaries, which both drain farmland and cultural landscapes. The substrates in both of the slowly flowing SW-tributaries consist of fine sand, gravel and smaller rocks. There are few, to none, large physical migration obstacles for the migrating grayling. The two SW-tributaries are connected to the north-western basin of the lake (Figure 1). This basin is known historically to have slightly higher nutrient level, and to be a little warmer compared to the other two basins found within the lake (Gammelsrud, 1982).

Hyrjon and Valåe, with their cold and swift currents, differ a lot from the two SW-tributaries. Both LC-tributaries drains from mountain areas, through steep slopes and birch (*Betula* spp.) and pine (*Pinus* spp.) forest areas. The tributaries have substrate consisting mainly of gravel and rocks with fine sand present near the outlets. There is no major

migration obstacles (i.e. water falls) found in the two LC-tributaries. Valåe is connected to the south-east basin of Lesjaskogsvatnet, and the outlet of Hyrjon is located in the junction area between the south-east and the mid basin (Figure 1).

Table 1: Summary of some environmental characteristics typical for the four sampled spawning tributaries in lake Lesjaskogsvatnet.

Tributary	Temperature regime	Mean tributary width (m)*	Flow regime
Søre Skotte	Warm	1.25	Slow flowing and laminar
Steinbekken	Warm	0.75	Slow flowing and laminar
Hyrjon	Cold	5	Fast flowing and turbulent
Valåe	Cold	6	Fast flowing and turbulent

* Mean tributary width is calculated (in meters) by Gregersen et al. (2008), based on measurements made on detailed maps.

Sampling

Temperature was measured every second hour in each tributary (except Steinbekken) using HOBO® loggers during the whole spawning period. The two LC-tributaries were very similar in temperature during the period mature grayling were observed in the respective tributaries (Figure 2). Søre Skotte proved to be warmer in the same period (Figure 2). Although no temperature measurements were available for Steinbekken in 2006, measurements done in 2005 and 2007 indicate that Steinbekken and Søre Skotte have highly correlated and similar temperature regimes ($r = 0.98$ with 8, and $r = 0.98$ with 109 measurements for 2005 and 2007 respectively. Linear relationship: $Y = -0.60944 + 1.0352 X$, where X is mean temperature in Søre Skotte at a given day, and Y is the mean temperature at the same day in Steinbekken).

Migrating fish were caught in fish traps. The fish traps used were designed not to harm the fish, but to entrap them and still enable them to move. The traps were put out prior to spawning and surveyed once, or twice, daily during the spawning run in each tributary.

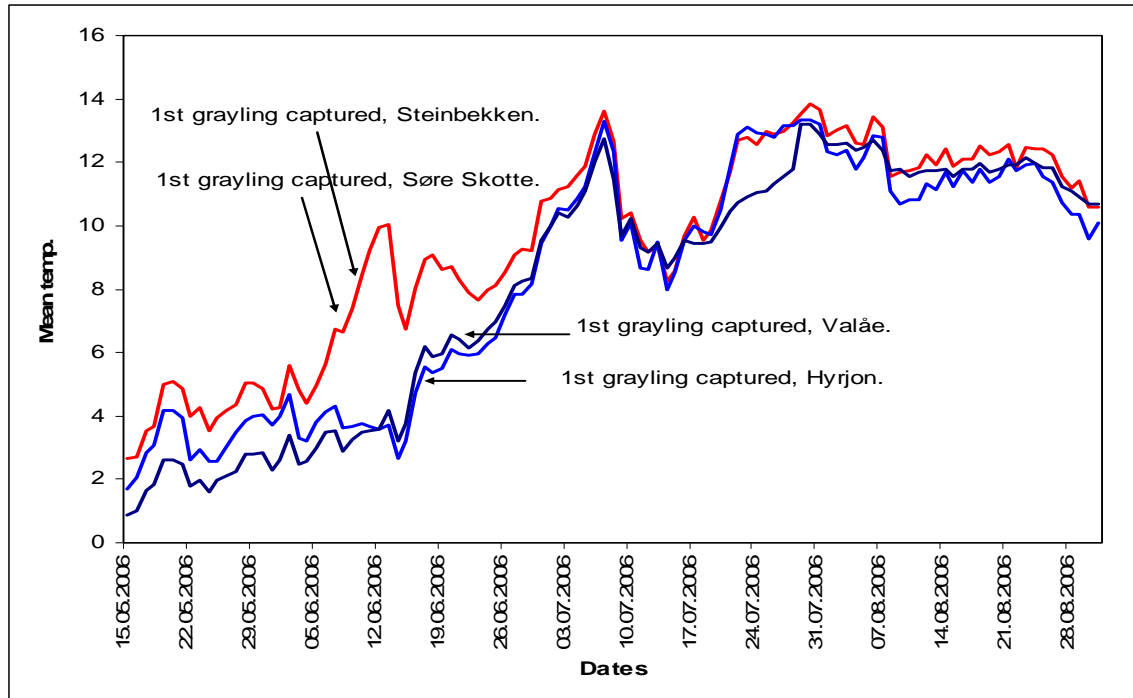


Figure 2: Mean water temperature trends for 3 tributaries, Sørskotte (red line), Valåe (dark blue line) and Hyrjon (light blue line), to Lake Lesjaskogsvatnet, 15.5-31.8 2006. Steinbekken is not shown by an individual trend. The temperature trend for Steinbekken is however calculated to be approximately similar to Søre Skotte. Arrows show when first spawning grayling was captured in the different tributaries.

In Søre Skotte and Steinbekken, the first spawning grayling individuals were captured the 8th and 11th of June 2006, respectively, while they were captured the 16th and 20th of June 2006 in Hyrjon and Valåe, respectively (Figure 2). We tried to intercept all migrating fish to get as complete a sample as possible. Traps and leading nets were therefore positioned to cover the whole width of the tributaries (Figure 3), but heavy flooding of the LC-tributaries hindered the use of traps here during shorter periods. Some of the grayling migrating past the traps in LC-tributaries were, however, later caught by electrofishing upstream the traps. Since most of the spawning fish were captured in all four tributaries, the total catches indicate the spawning stock size for the various demes in 2006.



Figure 3: Pictures of Steinbekken with fish trap (top left), Hyrjon with fish traps (top right), Søre Skotte with fish traps (bottom left) and a flooded Valåe (bottom right). In the large tributaries, fences were put out to lead fish to the fish traps. In the small tributaries the fish trap in it self blocked the whole tributary. The picture from Valåe is taken by Finn Gregersen.

When fish were observed in the traps (or electrofished), the captured fish were carefully transferred into larger tanks stationed in the respective tributary. The tanks had small ventilation holes, allowing for flow-through of natural water that secured good oxygenation and natural water temperatures. As soon as possible after capture, the individual grayling were sedated in a benzocaine solution. Fork length (in millimetres, mm) and mass (in grams, g) was then measured, sex determined and a sample of 5-10 scales taken for age determination. Following directly after this operation, the individual fish was photographed for morphometric measurements.

In order to standardize photo conditions in the field and to minimize fish handling and transportation, a specially designed transportable photo box was made. The camera was fixed at one position on top of the box, with a constant distance to the specimen (no camera zoom was used during photographing). Sun light was kept out with a covering

tarpaulin and by the box itself. The light conditions inside the photo box were held approximately constant, with a L.E.D. light and a flash bulb as the only light sources. The bottom of the box consists of polystyrene, with a drawn horizontal line and a measuring tape (see Figure 4). The sedated individual fish was placed along the horizontal line in the box, with the left side facing up. The dorsal fin was spread out to its maximum and fixed with small needles. A unique ID-label for each individual was also included in their respective picture.



Figure 4: A picture illustrating the placement of fish and the set-up, with an ID-mark and dorsal fin fixed with needles, within the photo box.

All pictures were taken using an Olympus μ 720 SW digital camera. Two independent pictures of each specimen were taken, where the best picture (i.e. best alignment of fish and best focus) was used for further analysis. The fish was then transferred to a recovery tank and allowed to recover after the handling, and then released to the tributary of capture, above the traps, to continue their spawning.

The original goal was to catch 100 specimens (50 males and 50 females) in every tributary. This goal turned out to be too optimistic. However, the number of fish caught in each tributary (see Table 2) should be statistically sufficient to get an estimate of the morphological variation (Svanbäck and Eklöv, 2002; Olsson et al., 2006). In all, a total of 355 individuals were caught and used in this study.

Ageing of the fish was done in the lab based on the scale samples (Hellawell, 1969). All scale samples used showed nice and clear annuli and were consequently easy to

interpret. Ageing was done twice independently, with the same outcome in 336 of the 355 specimens (94.6%), demonstrating the ageing method to be consistent.

Morphological analysis and statistics

General morphology was assessed based on the digital images. Landmark-based geometric morphometrics involving thin-plate spline (tps) analyses (Bookstein, 1989; Adams et al., 2004) were used to quantify morphological differences between specimens, sex and demes. This is a broadly used and easily interpreted method to assess a wide range of morphological data and aspects (e.g. Zelditch et al., 2004; Collyer et al., 2005 and references herein).

All the fish-photos were converted into one tps-file using tpsUtil (Rohlf, 2006c). It is then easy to import the tps-file, including all the photos, into tpsDig2 (Rohlf, 2006a). Here, 18 homologous landmarks were digitized, chosen to be able to visualize the overall body morphology on each specimen (Figure 5) and the x and y coordinates for each landmark were captured. The homology of the landmarks was first tested for by independently placing identical landmarks on the same fish 30 times and then measure the variation in each landmark configuration. This variation was found to be very low, $S^2 < 0.0095$ (calculated in tpsRelw (Rohlf, 2006b)), at every landmark configurations, indicating low human error in placing the landmarks homologous on every photo. The coordinates obtained from all the grayling in tpsDig2 were further analysed using tpsRelw. Non-shape variation (i.e. size) is here mathematically excluded, by performing a Generalized Procrustes Analysis (GPA) which, in short, scales the landmark configurations to a common unit size and then rotates the configuration to minimize the squared differences between corresponding landmarks (Adams et al., 2004). TpsRelw then produces a set of variables (partial warps and uniform scores) for each specimen which reflects the deviation in shape from the consensus specimen.

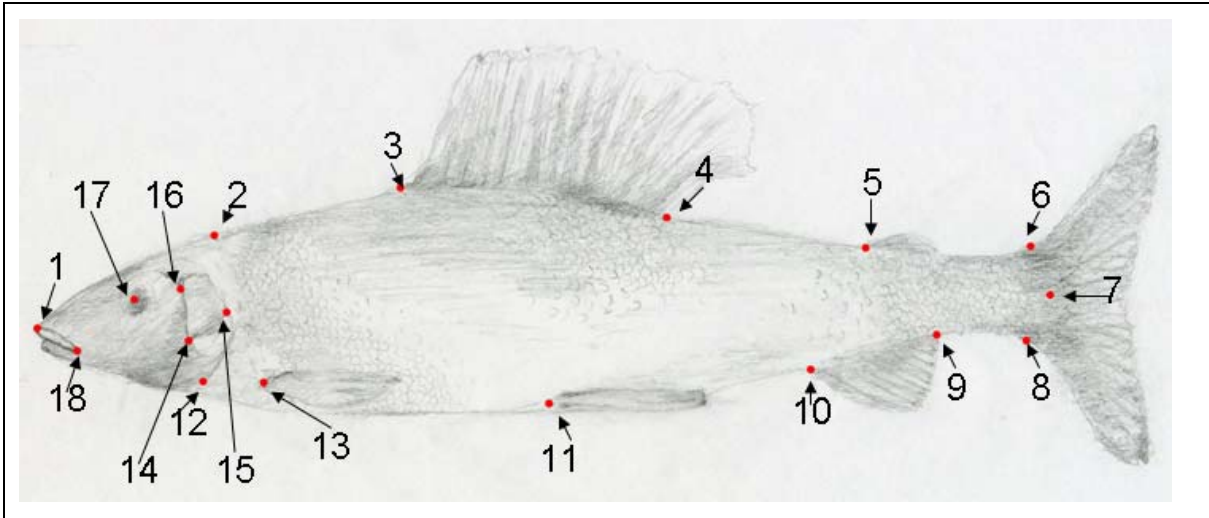


Figure 5: Positions of the 18 landmarks, with their corresponding numbers, digitized in tpsdig2 and used for morphological analysis of the grayling. Drawing is made by Andreas Bærum.

The individual partial warps (which describes local deformations) and uniform scores (shape changes over the whole body) can be treated as multivariate data representing shape, and be used for further statistical analyses (Svanbäck and Eklöv, 2003). To be able to visualize the morphological differences between tributaries, tpsRegr (Rohlf, 2007) was used. This program performs a multivariate multiple regression of shape onto the independent variables obtained from the statistics.

To get an indication of the dorsal fin size, the length of the 4th fin ray was measured. This was done on the digital pictures using linear measurements in pixels and then converted into millimetres (mm).

All statistical analyses were performed using JMP 5.0. To facilitate the interpretation of the morphological differences between specimens a discriminant analysis was used (Svanbäck and Eklöv, 2003). Gender plus tributary (each specimen was labelled with both gender and their respective tributary, this to effectively visualise both variables in one figure) was used as a grouping variable and the partial warps plus uniform scores as independent variables. This analysis maximally discriminates between the defined groups and produces a set of different functions, which each explains specific morphological deviations found in the sample. Each specimen is assigned with an individual canonical score for every general morphological variation recognised. A general morphological deviation in the data sample is represented as a function. Because of this, each canonical score given for the individual fish could be treated as a morphological value for the specific

function. The discriminant analysis also predicts the level of classification into the groups, based on how close the set of measured variables are to the means of the groups being predicted. Separate full factorial ANCOVAs were initially used to assess effects on each function and on the dorsal fin area. Here sex and tributary were included as factors. Length of the fish was used as a covariate to assess possible interactions of length on fish morphology. Insignificant interactions and effects (i.e. $P > 0.05$) were excluded from the different models as they appeared, and the model was then re-analyzed. This procedure was repeated until only significant effects were present. In addition, Tukey-Kramer honestly significant difference (HSD) tests were performed to test for morphological differences between sets of tributaries.

Results

Length, mass and age

There were significant differences between sexes in length, mass and age (two-way ANOVAs on each variable, all yielding a sex effect, all $P < 0.0001$) of the grayling sampled. There were, however, no significant differences between the tributaries, except for age (two-way ANOVA yielding a significant tributary effect on age, $F_{3,354} = 7.09$, $P < 0.0001$; for tributary effects on length and mass, both $P > 0.05$). Number of grayling caught, mean length, mean mass and mean age in the different tributaries are summarized in Table 2. Males were in general larger and older than females and the general grayling from Hyrjon was significant younger than the general grayling from the other tributaries (obtained from a Tukey-Kramer HSD-test, Alpha = 0.05).

Table 2: An overview of number of fish caught, mean length (\pm std), mean mass (\pm std) and mean age (\pm std) in the four sampled tributaries for females and males respectively.

Females				
Tributary	Sample size	Length	Mass	Age
Søre Skotte	94	295 (\pm 23)	261 (\pm 67)	4.8 (\pm 1.0)
Steinbekken	27	291 (\pm 20)	244 (\pm 57)	4.9 (\pm 0.8)
Hyrjon	16	283 (\pm 33)	227 (\pm 83)	4.1 (\pm 1.0)*
Valåe	38	299 (\pm 24)	257 (\pm 65)	5.0 (\pm 0.9)
Males				
Tributary	Sample size	Length	Mass	Age
Søre Skotte	100	324 (\pm 20)	314 (\pm 64)	5.6 (\pm 0,9)
Steinbekken	35	324 (\pm 22)	312 (\pm 64)	5.8 (\pm 0,8)
Hyrjon	11	314 (\pm 30)	280 (\pm 75)	4.5 (\pm 1,1)*
Valåe	34	319 (\pm 45)	303 (\pm 114)	5.3 (\pm 1,3)

* Shown, by a Tukey-Kramer HSD test, to be significantly different from all other tributaries.

Morphology

Seven functions, which together describe the total morphological variance in the sample, were yielded by the discriminant analysis (Table 3). The discriminant analysis correctly classified 72.7% of the 355 individuals to their respective “tributary x sex” group based upon the seven functions. The two first functions explain almost 77% of the total variation (Table 3). I therefore focused on the grouping, and the spread, of the canonical scores into these two different functions. The distribution of the mean canonical scores for each group, associated with the two functions, is visualised in Figure 6. The most evident divergence in the first functions is the deviation between sexes, but there seems to be differences between the tributaries as well. The second function gives the general impression that specimens from Valåe deviate from the three other demes, which in turn show some among-deme differences.

Table 3: Results of the seven morphological functions yielded by the discriminant analysis performed on the morphological differences between four demes of grayling in Lesjaskogsvatnet. Both males and females are included. The two first functions, in bold, explained the majority (76,8%) of the total morphological variation.

Morph. Function	Eigenvalue	Percent of total variance explained	Cum percent
1	2.581	56.3	56.3
2	0.937	20.5	76.8
3	0.480	10.5	87.3
4	0.232	5.1	92.4
5	0.173	3.8	96.1
6	0.098	2.1	98.3
7	0.077	1.7	100

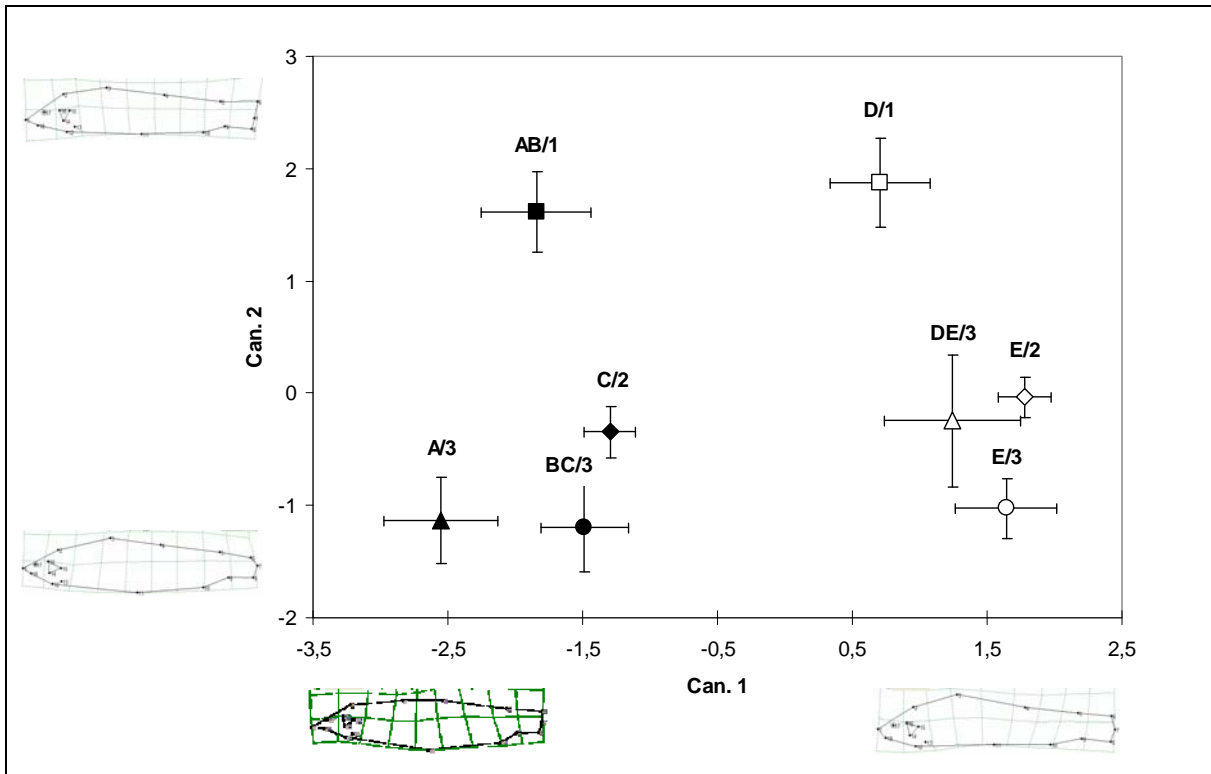


Figure 6: Plot of mean ($\pm 2 \times se$) canonical scores on functions 1 (Can. 1) and 2 (Can. 2) yielded by a discriminant analysis for the different groups. ▲ = Hyrjon, ■ = Valåe, ● = Steinbekken, ◆ = Sørskotte. Filled symbols represent females and unfilled represent males. Letters and numbers above each symbol represent the relationship (obtained by a Tukey HSD test) among the groups. Letters relates to the morphological differences found in the 1.function, and numbers to the morphological differences found the 2.function. Groups not sharing a common letter or number are significantly different from each other on the respective morphological function. Grid plots are graphical visualisations of the four extreme values for the two functions. Shape differences shown by the grid plot have been magnified 3 times to ease interpretation.

The first morphological function

The first morphological function, explaining 56.3% of the total variance, describes translocations mainly of landmark positions 1, 3, 4, 10, 11 and 18 (see landmark positions in Figure 3). This represents a deviation in the dorsal area; the abdomen region; angling of the maxilla bone; and the anal fin as viewed in tps-Regr (Figure 6 and Figure 7). The movement of landmark 3, 4, 10 and 11 are of greatest magnitude, and have thus largest impact on the morphological variance observed. Figure 6 also reveals a clear difference in mean canonical scores between males and females.

Males, which in general have been assigned with positive canonical scores (Figure 6), tend to have a powerful and elevated dorsal “hump” (Figure 7a). In contrast, the females, which in general have been assigned with low negative canonical scores (Figure 6), have a less expressed and more levelled dorsal hump (Figure 7b). In addition, males

tend to have a steeper angled maxilla bone and a longer proximal end of the dorsal fin compared to the females. Females show in general to have a more swollen abdomen than males, which is expected during spawning considering their large ovaries.

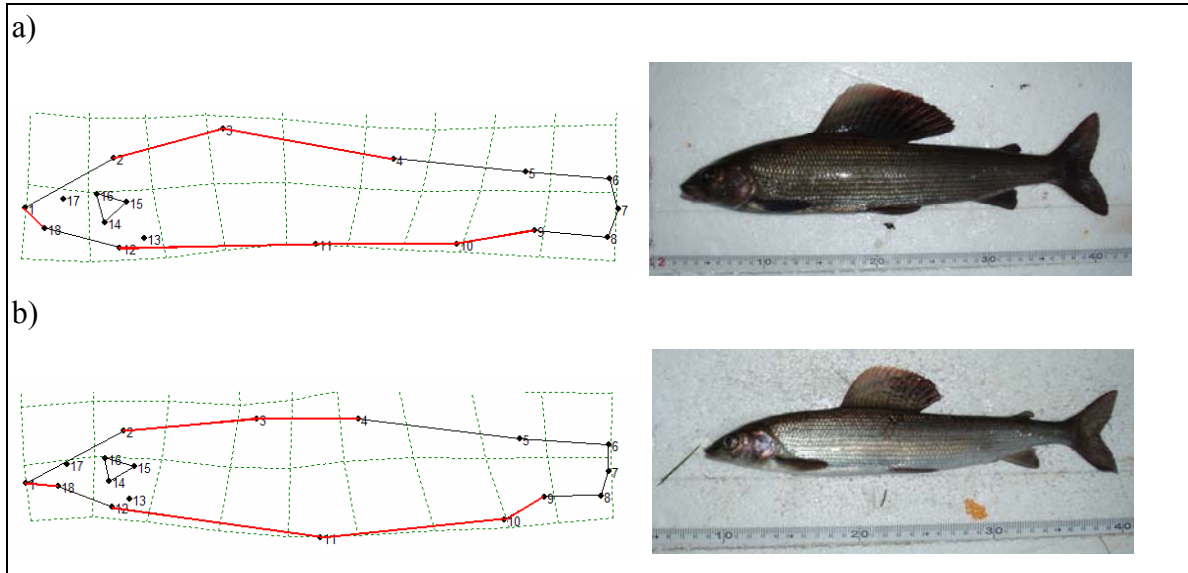


Figure 7: A closer look at the morphological deviation found in the first function. To the left, a visualisation (from tps-Regr) of the two extreme values for the first morphological function. a) represent positive scores, which is typically males, and b) represents negative scores (females). To the right, original pictures of the specimens expressing the extreme values. Red lines indicate areas with apparent morphological divergence. Grid plots are obtained from tpsRegr (Rohlf, 2007). The morphological variations in the grid plots are magnified 3 times to ease interpretation.

There were significant tributary effects on morphology within both sexes (Tabel 4). The males from Valåe differed significantly from the males from the SW-tributaries, having in general lower canonical scores (Tukey-Kramer HSD-test). Males from Hyrjon had canonical scores in between males from Valåe and the highly scored males from the SW-tributaries (Figure 6). The females from the SW-tributaries had higher canonical scores compared to females from the LC-tributaries. Females from Søre Skotte had significant higher scores compared to both the LC-tributaries, while females from Steinbekken had only significant higher scores when compared to Hyrjon (Tukey-Kramer HSD-test). Females from Valåe had scores in between females from the two SW-tributaries and females from Hyrjon which in general had the lowest scores.

Table 4: a): Summary of an ANCOVA of tributary and length effects on the canonical scores at the first function for male grayling. b): Summary of an ANOVA of a tributary effect on the canonical scores at the first function for female grayling.

a)

Source	DF	Sum of Squares	F Ratio	P
Tributary	3	24.54	10.36	<.0001
Length	1	41.99	53.20	<.0001
Error	172	138.12		
Total	179	210.60		

b)

Source	DF	Sum of Squares	F Ratio	P
Tributary	3	25.35	8.66	<.0001
Error	167	166.90		
Total	174	192.25		

There seemed also to be some small variations in the degree of sexual dimorphism among demes. The Søre Skotte deme and the Steinbekken deme had approximately similar differences between sexes with mean canonical distance of 3.0 and 3.1, respectively, between the sexes. The Hyrjon deme and the Valåe deme seemed respectively to have larger (3.9) and smaller (2.7) differences between males and females.

Male morphology, as measured by the first canonical scores from the first function, varied with fish size (Table 4; Figure 8), indicating a change in overall morphology with size. The length effect was the same in all four tributaries, as indicated by the non-significant interaction between tributary and length ($P > 0.05$)

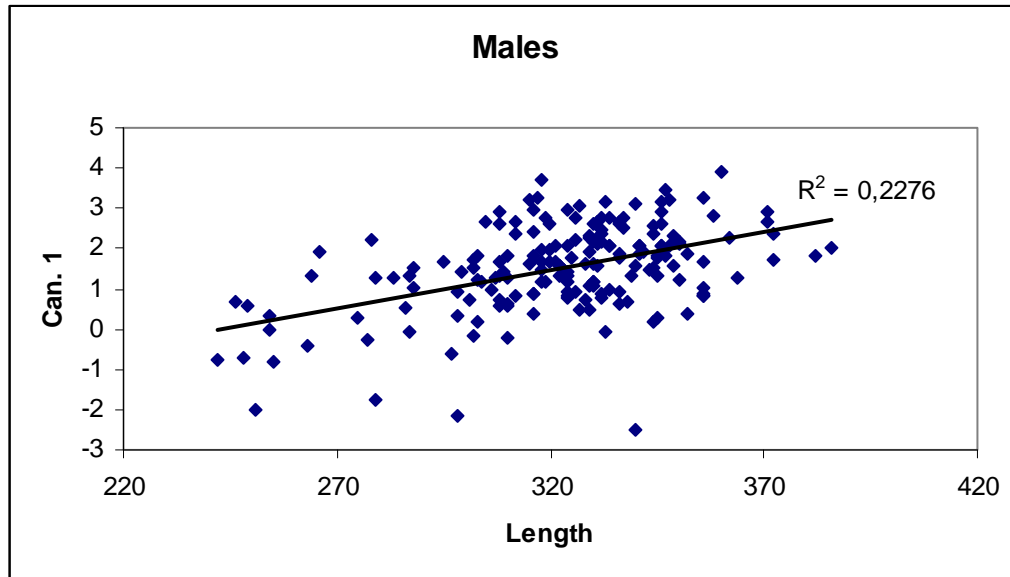


Figure 8: The relationship between length of the males and the canonical scores on the first morphological function. Fitted line represent the linear correlation ($R^2 = 0.23$ with $N = 180$, $P < 0.0001$).

The second morphological function

The second morphological function, explaining 20.5% of the total variance, shows a large variation in landmark positions 1-3, 6-9, 11-16 and 18 (see landmark positions in Figure 3). This represents a variation in body depth, head, operculum and tail morphology as visualised in Figure 9. Here, specimen with low canonical scores (i.e. below zero) tends to have a somewhat deeper midsection of their bodies compared to specimens with higher canonical scores (Figure 9). What is more visually obvious is the tendency for specimens with a low score to have a more or less dorsoventral symmetry anterior of the dorsal fin (Figure 9a). This is in contrast to specimens assigned with a high canonical score, which are more convex at their dorsal side compared to the ventral side, anterior of the dorsal fin (Figure 9b). The differences found in the shape of the operculum, seem to be linked to the overall divergence in head morphology. The difference observed in the tail region is mainly the movement of landmark 6 (see landmark positions in Figure 3). This makes the tail appear wider and more powerful whereas specimens assigned with a low canonical score obtain the apparently wider tail.

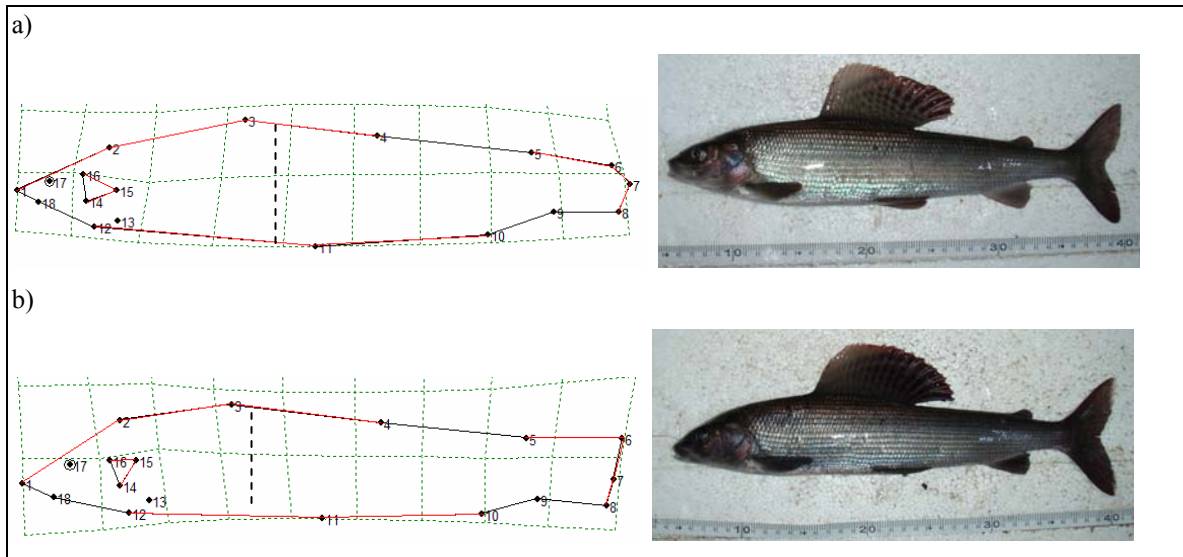


Figure 9: Visualisation of the morphological variation found in the second function. In a), the extreme low score is shown (left) with the corresponding picture (right). In b), the extreme high score is shown (left) and the related picture (right). Red lines indicate areas with clear morphological divergence. Left of broken lines are areas with differences in dorsoventral symmetry. Grid plots are obtained from tps-regr. The morphological variations in the grid plots are magnified 3 times to ease interpretation.

Specimens from Valåe tend to have higher canonical scores compared to specimens from either one of the other tributaries (Figure 6). However, there seems also to be significant differences among the grayling from the three other tributaries. This was confirmed by a two-way ANOVA (Table 5), yielding significant tributary and sex effect on morphology. A Tukey-Kramer HSD-test demonstrated specimens from Hyrjon and Steinbekken to be morphologically similar, with the overall lowest canonical scores (Figure 6). Specimens from Sørskotte have significant higher canonical scores than specimens from Hyrjon and Steinbekken, but significant lower scores compared to specimens from Valåe (Figure 6). Males tended to have somewhat higher canonical score (least sq mean: 0,08) compared to females (least sq mean: - 0,24).

Table 5: Summary of a two-way ANOVA used to quantify effects on variation in the second function.

Source	DF	Sum of squares	F Ratio	Prob > F
Sex	1	9.00	9.02	0.0029
Tributary	3	317.47	105.98	<.0001
Error	350	349.50		
Total	354	672.27		

The dorsal fin

There was a significant divergence in dorsal fin size between sexes ($F_{1, 252} = 48.612$, $P < .0001$), where males had larger dorsal fins compared to females (Figure 10). There was also a small, but significant interaction between sex and tributaries on dorsal fin size ($F_{3, 252} = 4.328$, $P = 0.005$). Separate ANCOVAs were thus used for each sex to assess the effect of tributaries on dorsal fin size (Table 6). Tributary effects explained more of the variation in fin size in females than in males. Length of fish had a large effect on fin size in both sexes, where larger fish had larger dorsal fins. A HSD-test revealed that males from Hyrjon had significantly smaller fin size compared to the other demes which had similarly sized dorsal fins (Figure 10). Females from Søre Skotte and Valåe proved to have significantly larger dorsal fins when compared to females from Steinbekken and Hyrjon (Figure 10).

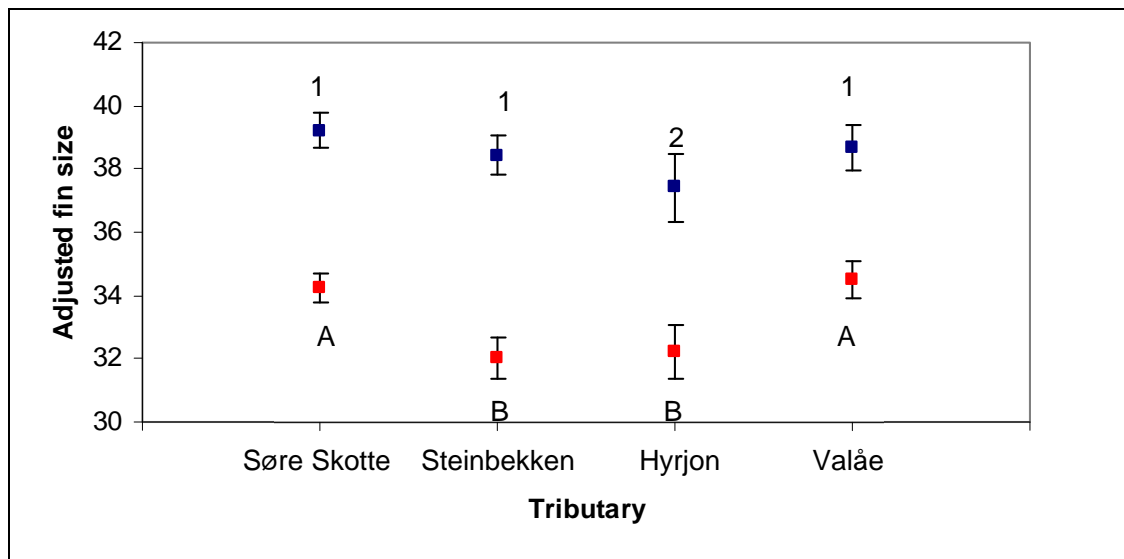


Figure 10: Mean dorsal fin size ($\pm 2se$), adjusted for length effect, for male grayling (blue) and female grayling (red) in the four sampled tributaries. Similar numbers for male grayling indicates similar sized fins, while non-similar numbers are significant different in size when compared to each other (Tukey-Kramer HSD-test). The same hold for females, only here in letters.

Table 6: Summary of two separate ANCOVAs on effects on dorsal fin size for males and females .

Males				
Source	DF	Sum of Squares	F Ratio	P
Tributary	3	32.53	3.34	0.022
Length	1	1014.22	311.96	<.0001
Error	112	364.12		
Total	116	1461.08		

Females				
Source	DF	Sum of Squares	F Ratio	P
Tributary	3	146.14	17.37	<.0001
Length	1	957.27	341.26	<.0001
Error	131	367.47		
Total	135	1647.58		

Discussion

In this study I have shown there to be significant differences in morphology between sexes and four demes in Lesjaskogsvatnet. The clear difference between sexes was seen both in general body morphology and also dorsal fin size. There were also differences between demes of these same traits. There seemed, however, not to be any large variation in the degree of sexual dimorphism among the demes. I also observed a second divergence among demes in body morphology. Here, especially one deme, the Valåe population, deviated from all the other demes in having significant different morphology. The morphological divergence observed could be an effect of sexual selection and/or natural selection.

Effect of sexual selection

Sexual dimorphism

There was a clear morphological difference between sexes in the sample. In general, males possessed a more dorsoventral angled **maxilla bone**; smaller **abdomen**; longer proximal end of the **anal fin**; and a larger **dorsal hump** and **dorsal fin** compared to females. The differences in these traits could be a result of either sexual selection or natural selection.

Biting among rivalling males is commonly observed at the spawning ground (Fabricius and Gustafson, 1955; Kratt and Smith, 1980). The more dorsoventrally angled **maxilla bone** observed in males could represent a response in the mouth morphology (e.g. larger or more powerful mouth) to a more specialized biting weapon. If this is so, the trait is likely to be selected for by sexual selection. Large elongated jaws, dedicated to male-male interactions, are commonly observed in salmonids (e.g. Tchernavin, 1938; Quinn and Foote, 1994). Deviation in mouth morphology is, however, also observed co-occurring with dietary variation in fishes (e.g. Norton, 1995; Wainwright and Richard, 1995; Wainwright, 1996; Frederich et al., 2008). This might suggest a functional response to variation in diet between males and females in the lake. There is however no evidence supporting or rejecting the latter.

Females have a larger **abdomen** than males. This is expected as female salmonids invest more heavily in gonad production than males (Hendry and Stearns, 2004; Quinn,

2005). This will lead to an overall divergence in morphology between sexes. However, these differences do not seem to be driven by sexual selection, but are rather driven by natural selection on egg size and fecundity.

The apparently wider, and more horizontally positioned, **anal fin** seen in males seems to have no obvious explanation in sexual selection. Nor has this been described, to my knowledge, as a sexual dimorphic trait in other salmonid species. It is therefore difficult to suggest a functional cause of a wider anal fin in males to other systems. It might prove useful as an extra stabilizing effect during the spawning act (Standen and Lauder, 2005). It could also be that inter-sexual variation in this trait is due to selection for a larger internal cavity in females, making room for more or larger eggs. Further investigation is needed to test the causalities of the inter-sexual variation in this trait.

The most prevailing morphological difference between the sexes is the enlarged males **dorsal hump** in male grayling. This type of sexual dimorphic trait is also seen in other salmonid species, as in *Oncorhynchus nerka* (Quinn and Foote, 1994), *Oncorhynchus tshawytscha* (Kinnison et al., 2003) and *Oncorhynchus gorbuscha* (Berg, 1979). This exaggerated trait is generally believed to be the result of both/either male-male competition (*reason 1*) and/or mate choice (*reason 2*) by females (Quinn, 2005). Although not as expressed as in some of its salmonid relatives, the male hump seen in grayling could have evolved for the same reasons.

Reason 1: Aggressive male-male encounters are common during the spawning period. When evenly matched in size, and when biting does not solve territorial conflicts, male competitors use their body to push each other away (Fabricius and Gustafson, 1955). This showdown could be done by a lateral display of size and fins, where the competitors try to force the opponent away sideways. It could also be done by one male swimming in front of the other and then using its lateral side to push the competitor downstream. A deep body, with a large hump, could in both cases prove to be of advantage.

Reason 2: Given the disparity between sexes in reproductive input, salmonid females are usually more selective of their mates than males (Hendry and Stearns, 2004; Quinn, 2005). A larger hump could give both better support (i.e. larger support muscles and tissue) for the important dorsal fin (Fabricius and Gustafson, 1955), and an overall impression of a powerful male. These traits might be favoured by choosy females.

The large **dorsal fin** in males could act both to attract females and to intimidate male competitors (Fabricius and Gustafson, 1955). It has also been observed to be folded over females during the spawning act (Fabricius and Gustafson, 1955; Kratt and Smith, 1980). The dorsal fin could therefore be subjected to both intra-sexual selection and inter-sexual selection.

Summarised, the morphological traits mentioned above, besides abdomen area and maybe also anal fin size, seem to represent adaptive secondary sexual characters due to sexual selection either by male-male interactions or by female choice. As sexual selected traits also are likely to be targets of natural selection (Svensson and Gosden, 2007), among-deme variation in these traits is most likely due to differences in the pressure of either of the two selection forces, or both combined.

Among-deme variation

There is a trend in the data that specimens from the LC-tributaries possess less expressed secondary sexual characters, except for dorsal fin size, compared to the specimens in the SW-tributaries. This observation may support the first prediction concerning H1; morphological variation due to increased strength of sexual selection in the SW-tributaries. It may, however, also indicate stronger natural selection against secondary sexual characters in the LC-tributaries, compared to the SW-tributaries, as the dorsal hump may increase the drag experienced. The latter support the prediction of H2. Both natural and sexual selection may thus favour the same expression of some of the secondary sexual characters in the respective tributaries. It may therefore be difficult to separate the two as to which gives the largest contribution to the morphological variation. Anyhow, I would expect, now in both hypothesized scenarios, to find reduced differences between the sexes in the LC-tributaries compared to the SW-tributaries. In the case of disparities in sexual and/or natural selection between tributaries, I would also intuitively expect the variation in secondary sexual traits to be larger in males (e.g. Berg, 1979; Hendry and Stearns, 2004; Quinn, 2005)

While there seems to be somewhat less sexual dimorphism in secondary characters in specimens from Valåe compared to specimens from the two SW-tributaries, the greatest sexual dimorphism is found in Hyrjon. The latter finding does not support the second

prediction of H1; more pronounced sexual dimorphism in the SW-tributaries. Neither does the observation support H2, as I here should expect males and females from both the LC-tributaries to have a more similar morphology (i.e. reduced dorsal hump in males). As a cautionary note, one should bear in mind that Hyrjon have a small sample size and might thus be biased. There was also observed an among-deme variation in dorsal fin size, which is likely the most important sexual secondary character in grayling (Fabricius and Gustafson, 1955; Kratt and Smith, 1980). Females from Søre Skotte and Valåe, and Steinbekken and Hyrjon have, respectively, similarly sized fins. Males do not express the same variation pattern. In fact, the general male from all tributaries, other than Hyrjon, seems to have a similarly sized dorsal fin. In the case of divergent strength of sexual selection, I would expect larger fins in the SW-tributaries (H1:P1). I would also expect the inter-deme variation in fin size to be larger among males compared to females (H1:P2). The variation seen in dorsal fin size seems thus not to support the hypothesis of morphological variation due to tributary differences in sexual selection gradients (H1).

It could be that there is a stabilizing selection (Brodie et al., 1995; Futuyma, 2005) for dorsal fin size in males. This would produce an optimal dorsal fin size relative to fish size within the lake and may be observed as an evolutionary stasis on the given trait (reviewed in Merilä et al., 2001). This assumption is supported by the observations of the small among-deme variation in fin size, and the strong correlation between male size and dorsal fin size. Although it seems unlikely that the trait is subjected to completely stasis, it may experience a slowly moving optimum, perhaps within genetic constraints obtained from the “ghost of the selection past” (Van de Peer et al., 2001; Estes and Arnold, 2007). This would lead to slow rates of evolution on this trait. Females may not be exposed to as strong a stabilizing selection on dorsal fin size as males. Hence, I observed a larger difference in female fin size among demes. The dorsal fin size may thus have a much lower divergent rate, compared to the other observed secondary sexual characters, not experiencing the same stabilizing selection. Females might show the same magnitude of variation as males in morphological traits because a natural selection agent, such as temperature, might impose a uniform effect on both sexes. Differential temperatures have been shown to affect differences in individual growth and development of grayling fry, with a potential genetic source of variation (Haugen, 2000; Krogstad, 2008). Thus,

deviation in the secondary sexual characters may not necessarily reflect adaptations to the different spawning environments, but rather occur as a side-effect of variations in early development and growth under disparate temperature and flow regimes (Sagnes et al., 1997; Sagnes et al., 2000). It may also be that there are large variations in other sexual cues connected to the dorsal fin, like colour. This is not measured in this study as true colours, and colour variations, are difficult to assess in the field. Colour is, however known to be of importance in sexual selection in different fishes (e.g. Endler, 1983; Seehausen and van Alphen, 1998; Boughman, 2001).

Another, and perhaps more likely, explanation of the among-demes variation in secondary sexual characters is due to founder effects in each tributary as they were colonized. The expression of secondary sexual characters in each deme might then have been reinforced by natal homing and thus reduced gene flow between tributaries. This might open for the possibility of phenotypic variation among demes as a result of genetic drift (Lande, 1976). The low morphological variation seen in secondary sexual characters may thus prove to have none, or very limited, fitness consequences in the different tributaries. This could account for all the trends seen in the data sample; the almost similar among-deme variation in both females and males; lack of a clear trend in phenotype x environment interactions; and the peculiar variation in the anal fin size and position.

Effect of natural selection

Grayling from Valåe possesses a more down-bent head and mouth compared to the other demes. They also seem to have a somewhat longer proximal end of dorsal fin and do diverge in tail morphology when compared to the three other demes. (*Caveat lector*: The landmark coordinates at the fish tail were difficult to make homologous on every photo, and may thus exhibit larger variation than actual). The morphological variation seen in the second function, bears thus a resemblance to the morphological differences observed in other systems with fish populations experiencing habitat structures (i.e. benthic/pelagic) and resource polymorphism (reviewed in Smith and Skúlason, 1996; Cussac et al., 1998; Dynes et al., 1999; Langerhans et al., 2003; Olsson and Eklöv, 2005; Olsson et al., 2006; Frederich et al., 2008; Svanbäck et al., 2008). This suggests that the differences seen could

be a result of resource competition, or variable resources, within the system. Both the mature grayling and the fry spend a short time in the natal tributary, and feed mainly in the lake. Consequently, it is difficult to link these findings to the diverging environmental conditions between tributaries. A more likely scenario could be an effect due to varying conditions in the different basins. Hence, this observation may suggest that the grayling spends more time in the basin connected to its natal tributary, compared to time spent in the other basins, weakening the hypothesis of uniform selective agents in the lake (H3).

In his MSc thesis in 1982, Gammelsrud conducted an extensive monitoring of different environmental factors of the different basins comprising Lesjaskogsvatnet. He found that graylings in the lake utilized both shallow (0-6 meters) and relatively deep waters (>10 meter) during the summertime. One explanation for utilizing the deep water was to avoid interspecific competition with brown trout. Valåe, which differs most from the other tributaries, drains into the south-east basin (Figure 1). This basin is also the deepest with a maximum depth of 34 meters compared to 14 and 19 meters in the other basins (Gammelsrud, 1982). In addition, Gammelsrud observed diverging prey (i.e. zooplankton etc.) composition among basins. Hence, these findings may help explain the observations of polymorphism within the lake, if the fish is constrained to different basins.

Caveats and limitations of the study

Experimental design

The two SW-tributaries are located in a different basin compared to the two LC-tributaries. The observation that the different basins within Lesjaskogsvatnet may vary in environmental conditions (Gammelsrud, 1982), and that the grayling may spend more time in one basin, complicates the exploration of among-deme variation. It is subsequently difficult to single-out various environmental conditions in the tributaries as the only selection agents affecting the morphological variation. A more ideal situation would have been to study morphological differences between LC-tributaries and SW-tributaries within the same basin to have more control on possible basin effects.

The use of geometric morphometric proved to be an effective method to assess the morphological patterns in the sample. The morphological variation observed is limited, however, to the difference in, and the number of, landmark configurations. There are also

further limitations on how many good homologous landmarks one could place on the grayling. Without prior knowledge of the true morphological variation, it might hence be difficult to correctly place the landmarks so that all important variation is captured. The landmarks 6-8 (Figure 5) proved difficult to place on some grayling individuals, as shadows and variation in colours made these spots difficult to define in some photos. This false variability in these landmark configurations may be observed as morphological variations in the tail region, without any factual roots. Rest of the morphological variability seen in this study is however based on more or less certain homologues landmarks. It should therefore represent factual morphological variations between demes.

Is statistical variation biologically relevant?

One important problem which remains unanswered is the question of biological significance. The inter-tributary divergence in morphology is of such small magnitude that it could hardly be seen by the eye. Nevertheless the discriminant analysis did correctly classify almost 73% of the grayling into their respective tributary. This high degree of correct classification gives a good indication that a morphological divergence between both tributaries and gender is present, and to be trusted. Do however the differences in morphology between tributaries represent any fitness related advantages? The differences in secondary sexual characters between demes are of a very small magnitude and may turn out not be irrelevant for fitness. However, the morphological difference between the basins is more visual and may well represent a biological relevant divergence. To truly enlighten these question one should test for fitness variations in the various environments between demes. This could be done in controlled common-garden experiments where one could vary variation in flow regime and density at the spawning ground and thus test for variation in fitness between demes. One could also explore prey capture success between demes on various prey associated with different habitats.

Local adaptation vs. plasticity

The difference in morphological traits seen may represent heritable local adaptations, phenotypic plasticity or a combination of both. Their relative importance as causal factors to the trait differentiation is an important issue in determining the possible contemporary evolutionary aspect. It is however difficult to determine the genetic basis of a trait by a

direct morphological study in the wild. Hence, one should do further studies of the morphology in common-garden experiments. This was unfortunately not possible within the time limits of this study, as common-garden experiments ideally use individuals from lineages reared in common environments for two or more generations. By doing so, one should be able to rule out differences between groups, caused by phenotypic plasticity and/or maternal effects, hence reveal possible genetic variation.

Summary and concluding comments

In this study, I found differences between demes in morphological traits recognized as secondary sexual characters. There are however difficult to pinpoint a common cause of the variation among demes in these sexual dimorphic traits. It may occur due to adaptation to different environmental conditions, or it may represent founder effects and genetic drift. The large male dorsal fin proved to be almost similar in size between the demes. This could be a result of fixed male-choice preferences in females, or the trait may be genetically constrained from selection past. The females are more variable in dorsal fin size.

The morphometric data also show differences in non-sexual traits resembling resource polymorphism in other systems. This difference between demes in the different basins is highly visual and may well prove to be biologically relevant. This variation might thus indicate that the lentic grayling spends more time in the basin where their natal spawning tributary is connected to. It also indicates that environmental conditions are likely to vary between the different basins within Lesjaskogsvatnet. Further studies are needed to account for local adaptations and phenotypic plasticity. Finally, the findings from this study should also be seen on as a part of the ongoing larger project: “The early stages of adaptive radiation: sympatric divergence in grayling”. With the now known morphological variation within the system, which presumably have occurred during only 20-25 generations, the cause of these variation may be more deeply explored. This might then help shed more light on the importance of processes behind contemporary microevolution and its importance in ecological speciation.

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